Zinc Deficient Bovine Erythrocyte Superoxide Dismutase Has Low Specific Activity

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Zinc deficient bovine superoxide dismutase (Cu_2E_2SOD (E = empty)) was prepared and purified by high performance liquid chromatography (HPLC). Each peak was characterized as to protein, copper content and specific activity. The Cu_2E_2SOD peak fractionated by HPLC has a low specific activity at pH 7.8 (about 10% of the native enzyme (Cu_2Zn_2SOD)). With the addition of zinc ions, the specific activity of Cu_2E_2SOD was quantitatively restored to that of the native enzyme. This behavior implies that the zinc ion is very important for the appearance of enzyme activity.

Keywords copper zinc superoxide dismutase; zinc deficient superoxide dismutase; high performance liquid chromatography; copper binding

Bovine erythrocyte superoxide dismutase (Cu_2Zn_2SOD[1]) has two identical subunits, each of which contain one copper(II) and one zinc(II) ion. The X-ray crystallographic analysis of bovine superoxide dismutase has been performed by Tainer *et al.* at 2 Å resolution.[2] The main feature of the metal binding region is such that copper and zinc ions coordinate to the same imidazole ring of the histidine 61 residue.[2] The bridging His 61 residue in turn plays an important role, because the bond between the copper ion and the bridging imidazole is disrupted when the copper(II) ion is reduced to a copper(I) ion by an O_2^- ion.[2,3] A proton then binds to the nitrogen atom of the disrupted imidazole in the His residue, and this proton on the imidazole may be utilized by O_2^{2-} in the substrate catalytic cycle.[2,3]

The copper ions at the copper-binding site are absolutely necessary for the enzyme activity.[3] Roe *et al.* proposed that the zinc ion is important for stabilizing the conformation of the enzyme.[4] Fee *et al.*[5] proposed that zinc deficient bovine erythrocyte superoxide dismutase (Cu_2E_2SOD) has almost the same activity as that of the native enzyme (Cu_2Zn_2SOD) at pD 9.3 using pulse-radiolysis analysis. Recently, Bertini *et al.*[6] showed that Cu_2E_2SOD derivatives, both wild types and mutants, have low activity at a physiological pH of 7.4. In literature,[5-9] the specific activity of Cu_2E_2SOD varies widely, from 20% to 80%. Metal coordination at the zinc site would be expected to facilitate deprotonation from the disrupted His 61 residue.[2,3] However, the role of the zinc ion is not yet clear.

In the previous paper,[10] $Cu_2CuESOD$ and Cu_2E_2SOD could not be purified by high performance liquid chromatography (HPLC). But in this paper, $Cu_2CuESOD$ and Cu_2E_2SOD purified by HPLC were characterized, and the role of zinc ions in the native enzyme was discussed.

Materials and Methods

Chemicals and Reagents Bovine superoxide dismutase (Cu_2Zn_2SOD) was purified from bovine erythrocytes using the method of McCord and Fridovich.[11] The apo-bovine superoxide dismutase (E_2E_2SOD) (E = empty) was prepared by dialysis against ethylenediaminetetraacetic acid (EDTA) (10^{-3} M) at pH 3.8 (0.01 M acetate buffer) and then 0.1 M sodium chloride (0.01 M acetate buffer) to remove excess EDTA.[10] The zinc-free bovine superoxide dismutase (Cu_2E_2SOD) was prepared by two different methods: method I, the copper ions were slowly added to E_2E_2SOD at a $[Cu^{2+}]/[E_2E_2SOD]$ ratio of 2.0 in 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5)[8,9]; method II, the native enzyme (Cu_2Zn_2SOD) was dialyzed by 0.01 M acetate buffer (pH 3.8).[12] In the latter method, the zinc ions in the native enzyme were selectively removed.

Apparatus and Conditions for HPLC The copper and zinc content was measured with Shimadzu AA-630-12 flame and AA-670G flameless atomic absorption spectrophotometers.

A high-performance liqiud charomatograph (Shimadzu LC-3A) was equipped with a TSK-gel DEAE-2SW column (4.6 i.d. × 250 mm) connected to its precolumn (4.6 i.d. × 50 mm) and an ultraviolet (UV) detector (Shimadzu SPD-2A; at 260 nm). The elution system (flow rate, 0.5 ml/min) had a linear gradient from 0.006 M K_2HPO_4–HCl buffer (pH 7.0) containing 10^{-4} M ethylenediaminediacetic acid (EDDA) to a 0.05 M K_2HPO_4–HCl buffer (pH 7.0) containing 10^{-4} M EDDA (gradient rate; 0.5%/min).[10] Twenty μl of the enzyme solution (about 10^{-4} M) was injected into the HPLC column. The enzyme activity was measured immediately after fractionation, then the fractionated solution was concentrated and dialyzed against metal-free distilled water to measure the metal content of the enzyme.

Enzyme Activity and Protein Concentration The enzyme activity was measured at pH 7.8 with 0.1 mM EDTA (McCord and Fridovich[11]). The Shimadzu UV-200 spectrophotometer was thermostated at 25 ± 0.5 °C. The concentration of protein was determined by the method of Lowry, with bovine serum albumin as a standard.

Results

The HPLC chromatograms of Cu_2E_2SOD obtained by methods I and II as described in the Materials and Methods section are shown in Fig. 1. The HPLC chromatogram of Cu_2E_2SOD obtained by method I has one small peak and two main peaks (Fig. 1). In order to characterize peaks 1, 2 and 3, these peaks were fractionated and copper, protein content, and specific activity of each peak was determined (Table I). In Table I, peak 3 can be assigned to Cu_2Cu_2SOD on the basis of its copper content (3.81/enzyme) and high specific activity (4100 unit/mg). The retention time and specific activity of peak 3 is completely consistent with that of Cu_2Cu_2SOD prepared by the method of the literature.[10] The copper content of peak 2 was

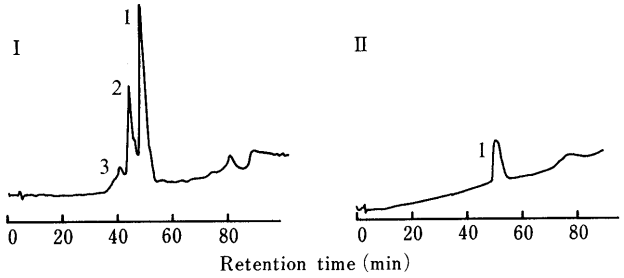

Fig. 1. Chromatograms Illustrating the Separation of Cu_2E_2SOD Prepared by Methods I and II

I: Cu_2E_2SOD prepared by method I. II: Cu_2E_2SOD prepared by method II.

TABLE I. Copper Content and Specific Activity (pH 7.8) of Each Peak Fractionated by HPLC in Cu_2E_2SOD Obtained by Methods I and II

	Method I			Method II
	Peak 1	Peak 2	Peak 3	Peak 1
Protein (M)	3.12×10^{-5}	1.56×10^{-5}	0.80×10^{-5}	3.9×10^{-5}
Copper (M)	5.90×10^{-5}	4.26×10^{-5}	3.05×10^{-5}	6.2×10^{-5}
$[Cu^{2+}]$/[enzyme]	1.89	2.73	3.81	1.60
Activity (Units/mg of protein)	546	2235	4100	515

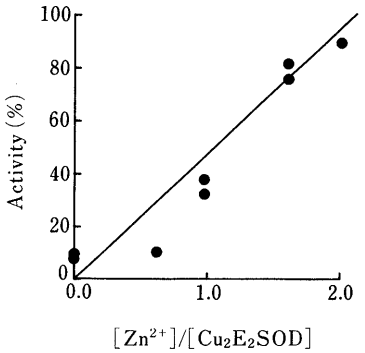

Fig. 2. Recovery of Enzyme Activity by Adding Various Amounts of Zinc Ions to Cu_2E_2SOD

Various concentrations of the zinc ion were added to 1.0×10^{-4} M Cu_2E_2SOD at pH 7.4. Enzyme activity was measured by the xanthine–xanthine oxidase method at pH 7.8.[11]

2.73 copper ion per enzyme and the specific activity was about a half of Cu_2Cu_2SOD. Therefore, peak 2 must be $Cu_2CuESOD$. The copper content of peak 1 was 1.89 Cu per enzyme molecule, but it had very low specific activity (546 unit/mg). From its copper content, peak 1 will be assigned to Cu_2E_2SOD. On the basis of these results, it is indicated that Cu_2E_2SOD prepared by method I is a mixture of Cu_2Cu_2SOD, $Cu_2CuESOD$, and Cu_2E_2SOD. Thus, method I is not a good method for preparing Cu_2E_2SOD. Surprisingly, Cu_2E_2SOD fractionated by HPLC has very low specific activity at pH 7.8 (about 12% of the native enzyme (4500 unit/mg)). The specific activity of $Cu_2CuESOD$ is equal to half the specific activity of Cu_2Cu_2SOD (Cu_2Cu_2SOD has the same activity as that of Cu_2Zn_2SOD[10]). The metal binding to the zinc site is very important to the appearance of the enzyme activity.

In Fig. 1, the chromatogram of Cu_2E_2SOD obtained by method II has only one peak. This peak was also fractionated and its copper, protein content, and specific enzyme activity were measured. These values are also shown in Table I. The copper content of this peak was 1.6 atoms per enzyme. The specific activity of this peak was 515 units per mg of protein and is almost consistent with that of Cu_2E_2SOD prepared by method I and purified by HPLC. Method II is much better for preparing Cu_2E_2SOD than method I.

To make sure that Cu_2E_2SOD has very low specific activity, the recovery of enzyme activity following the addition of various concentrations of zinc ions to Cu_2E_2SOD obtained by method II is shown in Fig. 2. With the addition of zinc ions, the specific activity of Cu_2E_2SOD was quantitatively restored from 10% to 95%. At a $[Zn^{2+}]/[Cu_2E_2SOD]$ ratio of 2.0, the specific activity of

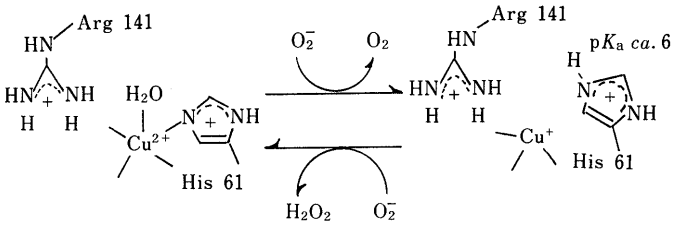

Chart 1. Reaction Mechanism of Cu_2E_2SOD in the Catalytic Cycle

Cu_2E_2SOD completely recovered to that of Cu_2Zn_2SOD. This behavior clearly indicates that Cu_2E_2SOD has very low activity (about 10% of Cu_2Zn_2SOD), but easily becomes Cu_2Zn_2SOD with the addition of zinc ions.

In a previous paper,[13] it was shown that the cupric ion of Cu_2Zn_2SOD was completely reduced by $Fe(CN)_6^{4-}$ (10^{-2} M) at pH 6.0. To know whether the redox potential of the copper ion in Cu_2E_2SOD is different from that of Cu_2Zn_2SOD, reduction of the cupric ion of Cu_2E_2SOD by $Fe(CN)_6^{4-}$ (10^{-2} M) was attempted. The cupric ions of Cu_2E_2SOD were not reduced by 10^{-2} M $Fe(CN)_6^{4-}$. But, with the addition of zinc ions (10^{-3} M) to Cu_2E_2SOD, the cupric ions in Cu_2E_2SOD can be reduced by $Fe(CN)_6^{4-}$, because the zinc ion is bound to the zinc sites in Cu_2E_2SOD. This behavior may imply that the redox potential of the cupric ions in Cu_2E_2SOD is much lower than in Cu_2Zn_2SOD.

Discussion

In Table I, Cu_2E_2SOD has very low specific activity. This behavior indicates that the zinc ion is very important for the appearance of enzyme activity. Why does the zinc ion accelerate the catalysis of superoxide ions?

In the native enzyme, the proton of the nitrogen atom of the disrupted imidazole in His 61 is supplied to O_2^{2-} in the substrate catalytic cycle (in Chart 1). The same reaction will occur in Cu_2E_2SOD. But, the pK_a of the histidine residue detached from cuprous ions that are 6—7 and 14 are so different from that ($pK_a = 10-11$)[3] of the native enzyme, that a proton of His 61 cannot easily be supplied to O_2^{2-} in the substrate catalytic cycle.[3] This may be the reason Cu_2E_2SOD has low specific enzyme activity.

Another explanation is also possible. In the experiment concerning the reaction between Cu_2E_2SOD and $Fe(CN)_6^{4-}$, the copper ion of Cu_2E_2SOD is not reduced by $Fe(CN)_6^{4-}$. This behavior may imply that the redox potential of the cupric ion in Cu_2E_2SOD is much lower than that in Cu_2Zn_2SOD. The change of the redox potential of the copper ion in Cu_2E_2SOD may be correlated to the low activity of Cu_2E_2SOD.

References and Notes

1) Cu_2Zn_2SOD is a native superoxide dismutase which has two copper ions in the copper binding sites and two zinc ions in the zinc binding sites. E_2E_2SOD represents the enzyme which has no metal ions (E indicates empty). Cu_2Cu_2SOD is the enzyme which has four copper ions in the copper and zinc binding sites. $Cu_2CuESOD$ is the enzyme which has two copper ions in the copper sites and one copper ion in the zinc sites. Cu_2E_2SOD is the enzyme which has two copper ions in the copper sites.
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